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Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates

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Abstract

Accurate detection of organisms is crucial for effective management of threatened and invasive species because false detections directly affect implementation of management actions. The use of environmental DNA (eDNA) as a species detection tool is in a rapid development stage, however, concerns about accurate detections using eDNA have been raised. We evaluated the effect of sampled water volume (0.25 to 2 L) on the detection rate for three macroinvertebrate species. Additionally, we tested, depending on the sampled water volume, what amount of total extracted DNA should be screened in order to reduce uncertainty in detections. We found that all three species were detected in all volumes of water. Surprisingly, however, only one species had a positive relationship between an increased sample volume and an increase in the detection rate. We conclude that the optimal sample volume may depend on the species-habitat combination and should be tested for the system where management actions are warranted. Nevertheless, we minimally recommend sampling water volumes of 1 L and screening at least 14 µL of extracted eDNA for each sample to reduce uncertainty in detections when studying macroinvertebrates in rivers and using our molecular workflow.

Introduction

By shedding hairs, cells, gametes or feces, all organisms leave traces of their occurrence in the environment in the form of so-called environmental DNA (eDNA). Recent reviews illustrate that many eukaryotes, including plants and animals, are readily and non-invasively detected from traces of their DNA found in water, soil and air.[1,2] The utility of this non-invasive molecular method for species detection has large implications for environmental management actions[3] and subsequent policy and stakeholder decisions e.g.,[4].

The method of detecting macro-eukaryotic species from traces of their DNA in the environment is, however, in a rapid development phase.[1,3] Experimental evidence of the power, but also the limitations of the tool is greatly needed in order to effectively track organisms in their environment from eDNA. Of particular concern are false negative detections, for example see[5,6,7]. False negative detection, or process type II error, in terms of environmental DNA, means that there is no DNA detected although the species is present at the sampled location (please note that false negative detections can also occur during "classic sampling", see for example[8]). False negative detections may be of particular concern for invasive species and species threatened with extinction because not detecting the species can have severe consequences for management decisions. It is therefore paramount that we gain a better understanding of the causes of false negative detections with eDNA such that it can be established as a viable and defensible method for species detection.[2,9]

Reasonable work has already focused on the causes of false negative detections and the possible means by which false negatives can be reduced. Causes of false negatives are due to the inherent problem of the detection limits of the molecular technology used to capture, extract and amplify the DNA found in the environment[10-13] but, in the case of meta-barcoding approaches,

61 also due to limitations of reference databases. The latter are not discussed here further, as we
62 focus on a targeted approach, but see for example[14,15]. Far less attention, however, has been paid
63 on estimating how sampling bias affects the detection rate for a species' eDNA and subsequently
64 resulting in false negative detections. Any biological sample suffers from a sampling bias based
65 on the probability of detection and is governed by the method(s) used for detection.[16]
66 Environmental DNA detection is no exception and has mainly two steps which can cause a
67 sampling bias either in the field and/or in the lab.[5] The first sampling bias can happen when a
68 particular amount of the environmental sample is collected, such as a set volume of air, soil or
69 water. The second sampling bias can happen when amplifying the targeted species' DNA from a
70 small fraction of the total purified DNA contained in the sample. Both of these steps involve sub-
71 sampling the potential pool of DNA that is tested and can result in a false negative detection
72 simply due to sampling error.

73 Focusing on freshwater, there are two possible environments to collect DNA from,
74 namely from the sediment or from the water column. Here we focus on estimating detection rates
75 from water, because DNA extracted from water has been commonly used in many macro-
76 organisms' detection protocols (Table 1). Water-based samples are thought to reflect the
77 contemporary, regional community of macroinvertebrates, while sediment-based samples are
78 more likely reflecting local communities, possibly integrating over time. It was not the goal of
79 our study to compare these two methods, as the methods differ and are covering different,
80 complementing aspects. For a comparison and discussion of sediment-based samples, see for
81 example[5,17].

82 In a water-based approach, a first sampling bias can be introduced when different sets of
83 water volumes are used. Likely, the volume of water sampled in different studies (for an

84 overview on volumes used in previous studies using filtration, see Table 1) has been a choice of
85 practicality based on the specific field and molecular protocol used to capture and concentrate the
86 eDNA (i.e., the logistical aspects of sampling) and is not necessarily a reflection of the optimal
87 amount needed to reduce uncertainty in the detection rate. For example, when eDNA is captured
88 from freshwater through precipitation, usually 15 mL of water is used due to the limitation of
89 centrifuge size needed for the next step to process the water in most standard molecular
90 laboratories. Filtration from freshwater as a capture method is more flexible with respect to
91 volume and previous studies have thus used volumes ranging from 100 mL[18] up to 10 L[19], with
92 an average of approximately 2 L (Table 1). Thus, when left to interpretation and method choice,
93 one could justify to sample and filter 100 mL to 10 L, but it is unclear if and how the volume of
94 water sampled affects the detection rate for a species.

95 A second sampling bias can occur at the polymerase chain reaction (PCR) stage, where a
96 wide range of total volume of extracted eDNA screened for targeted DNA has been used (Table
97 1). This is further confounded by the fact that varying molecular protocols have been used for
98 purification of DNA from freshwater, such that the total DNA screened in addition to the total
99 eDNA recovered from the sampled water is also a possible confounding factor.[10] Surprisingly,
100 we have no clear evidence pointing to an optimal amount of extracted eDNA needed to reduce
101 false negative detections (Table 1). For example, Goldberg *et al.*[19] screened 1 µL (but extracted
102 DNA from 10 L), while Wilcox *et al.*[12] screened 128 µL of their DNA extraction (Table 1). It is
103 known, that the PCR has an inherent stochastic component, which plays a major role when DNA
104 concentrations are low. As this is typical for extracted DNA from environmental samples, the
105 stochastic component of PCR needs to be considered when performing PCR on eDNA. Overall, it
106 is not completely clear how much volume of the total extracted DNA should be screened to have
107 a precise estimate of the effect on the detection rate for a species.

In this study we tested the effect of sampling at these two stages and how it affects false negative detections. Specifically, we sampled DNA from the environment in different volumes of water and then analyzed different volumes of DNA extracted from a given amount of water. We sampled independent volumes of water ranging from 250 mL to 2000 mL and tested for the detection of three macroinvertebrate species belonging to the orders of Mollusca, Ephemeroptera and Amphipoda at a location in a river where all three species are known to be present. We compared the detection rate with respect to the volume of sampled water and the volume of extracted DNA that was screened. We conclude with recommendations for optimal volumes of water to sample and how much volume of extracted DNA to screen in order to reduce false negative detections. Our recommendations refer to a similar set of species and study systems (i.e., macroinvertebrates in rivers). For other species or habitats they may be used as first guiding values.

Material and Methods

Field sampling

Our study site was located at the river Glatt (47° 26' 35.21" N, 8° 33' 03.94" E). It is a natural river belonging to the headwaters of the river Rhine catchment in Switzerland (for pictures of the study river, see[20]). We sampled water on September 17th and 30th in 2014. These sampling dates reflect classic sampling time points for macroinvertebrate (commonly done either in spring and/or fall). We sampled at two time points to avoid spurious effects due to a given day's hydrological regime, but close enough to each other to avoid changes in communities looked at. On each day we sampled two replicates of each of the following volumes: 250 mL, 500 mL, 1000 mL, 1500 mL and 2000 mL. This range of volumes was chosen based on previous work (see Table 1) to be suitable for our habitat, that is, freshwater streams and macroinvertebrates, and is

reflecting most of previous volumes considered. We sampled each volume independently in one or two individual 1-L sterile octagonal polyethylene terephthalate bottles (VWR International, Radnor, Pennsylvania, USA) that were previously decontaminated with 10% household bleach, rinsed with Milli-Q® (Merck Millipore, EMD Millipore Co., Billerica, Massachusetts, USA) water and exposed to ultraviolet C light (UVC) and sealed in a DNA clean lab in order to remove all possible contaminants of DNA. We collected surface water from the edge of the river and filtered each volume on site. For each of the sampled volumes we sequentially filtered batches of 250 mL of water onto a single 25 mm 0.70-µm glass fiber filter (GF/F, Whatman International Ltd., Maidstone, UK). The total number of filters used for each volume class ranged from one to eight filters. The filters were housed in a 25 mm filter case (Swinnex, EMD Millipore Co., Billerica, Massachusetts, USA) that was attached to a disposable 50-mL syringe. For each volume class we used the same filter housing and the same syringe and changed only the filter as necessary to process the total volume. After filtration, we transferred the filters into individual 1.5 mL tubes containing tissue lysis buffer (100 mM Tris-HCL pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM $NaCl_2$) using tweezers that were decontaminated with 10% household beach between volume replicates and rinsed with ethanol. The tubes were immediately stored on ice. The time between the first and last sample was approximately 2.5 h during field filtration. Samples were immediately transported to the laboratory with a maximum travel time of 30 minutes. Additionally, we created two negative filtration controls, which consisted of decontaminated Milli-Q® water treated with UVC light and sealed in a DNA free laboratory. We brought this water to the field and filtered 2000 mL before environmental samples were taken on each sampling day, resulting in 4 negative filtration controls. Upon return to the laboratory we immediately began the extraction as described below.

DNA extraction and species eDNA amplification

156 In the laboratory we performed a modified cell lysis phenol-chloroform-isoamyl extraction on
157 each single filter as this has been shown as an effective extraction method for eDNA from glass
158 fiber filters.[10] We added for each set of extraction a negative control (further called negative
159 extraction control). All eDNA extractions of each target volume were subsequently pooled, such
160 that eDNA was resuspended across all filtration volumes in a total volume of 100 µL. For
161 example, the two filters used for the 500 mL volume were each resuspended in 50 µL and then
162 pooled to equal the total extracted DNA volume of 100 µL. All pooled DNA extractions were
163 cleaned with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, California,
164 USA) according to the provided protocol. To countervail for volume loss during clean-up we
165 added 50 µL of AE buffer (this is the elution buffer from DNeasy Blood & Tissue kit delivered
166 from Qiagen GmbH, Hilden Germany) to each cleaned DNA extraction to allow for testing of
167 multiple species with the same extraction. Cleaning eDNA with this additional step has been
168 shown to be effective for removal of PCR inhibition of riverine samples of environmental DNA[13]
169 and we did not want PCR inhibition to additionally confound the detection rate. All pooled and
170 cleaned extractions were quantified by using the Qubit (1.0) fluorometer following the
171 recommended protocol for the high sensitivity (HS) assay for dsDNA (Life Technologies,
172 Carlsbad, CA, USA) and can be consulted in Figure S1.

173 We conducted standard PCR, because the primers used in this study were designed for standard
174 PCR for a previous study,[21] and thus allows comparability. Additionally we wanted to use a
175 method that seems commonly available to federal offices. We then performed eight PCR
176 replicates, where each replicate screened 2 µL with concentrations ranging from 0.153 to 3.73
177 µg/mL of extracted DNA, equaling 16 µL screened for each of the following three target species:
178 *Ancylus fluviatilis* (Mollusca)*, Baetis buceratus* (Ephemeroptera) and *Gammarus pulex*
179 (Amphipoda). Based on previous studies, we know that these species belong to the regional

180 species pool at this site from long-term monitoring data (1995 to 2012) provided by the Canton of
181 Zurich and our own sampling.[22-24] All negative filtration controls and all negative extraction
182 controls were tested individually in eight PCR replicates for the presence of each species. We
183 used primer probes previously designed and tested for eDNA detection of these species at the
184 study site.[21] For each PCR run we added a negative PCR control by adding molecular grade DNA
185 free water (Sigma-Aldrich, Co. LLC. St. Lewis, MO, USA) as template and a positive PCR
186 control (using tissue extracted DNA from the target species as PCR template). The PCR
187 components and thermocycling temperature were performed exactly as described in Mächler *et*
188 *al.*[21] and can be found in the supplementary information of this paper (S1). All PCR products
189 were visualized by electrophoresis on a 1.4% agarose gel stained with peqGreen (VWR
190 International, Radnor, Pennsylvania, USA).

191 From each volume of water we confirmed at least one positive PCR reaction by using Sanger
192 sequencing. We cleaned the PCR product with Exo I Nuclease (EXO I) and Shrimp Alkaline
193 Phosphatase (SAP) (Thermo Fischer Scientific, Waltham, Maryland, USA) as described in
194 Mächler *et al.*[21]. Sequencing was performed in both directions with BigDye® Terminator
195 (version 3.1) system on an ABI 3730xl. The software Sequencher® version 4.9 (Gene Codes,
196 Ann Arbor, Michigan, USA) was used to align, edit and compare our sequences with previous
197 eDNA sequences obtained from this site in a previous year and tissue derived sequences.[21] We
198 used the same criteria for a positive detection (band present on a gel and sequence confirmation
199 for each experimental volume) and rigorous laboratory precautions as described in Mächler *et*
200 *al*.[21] by creating, in addition to our negative filtration controls, negative controls for extraction
201 and PCR. In total we screened four negative filtration, two negative extraction and twelve
202 negative PCR controls for potential contamination.

Analysis

We analyzed the detection rate of each individual species with generalized linear models (GLMM). Volume of water was used as predictor variable and detection rate as a binary response variable consisting of the number of positive and negative detections out of the eight PCR replications. We used the two replicates per volume on a single day as a random effect, nested within the sampling day for *G. pulex* and *B. buceratus*. For *A. fluviatilis,* we had only one sampling day, due to a contamination on the first day.

We tested how the uncertainty in detection rates changed as a result of increasing the amount of extracted DNA screened for each species, using a resampling approach (bootstrap approach) of the individual assessments. We sampled 10,000 outcomes in detection rates from our data when screening 2 to 16 µL of DNA. As a measure of uncertainty, we subsequently calculated the median range (absolute difference between minimum and maximum) in detection rates over all outcomes for a given sample volume and species (data from the two sampling days were pooled). When uncertainty is one, detection rates can be any value between 0 and 1, that is, the estimate is uninformative; when uncertainty is zero, then the detection rate is the same for all outcomes and is maximally informative. All statistical analysis were done in R version 2.15.3 (R Development Core Team 2014)[25], and the package "lme4"[26].

Results

All three species were detected at the sampling site by the use of eDNA in all volumes and on both sampling dates (Fig. 1). We found a positive, significant relationship between sampling

224 volume and detection rate for *G. pulex* ($p < 0.05$, Table 2). For the other two species there was no
225 significant relationship (*A. fluviatilis* $p = 0.78$, *B. buceratus* $p = 0.72$, Table 2).

226 We showed for all three species that the uncertainty in the detection rate decreases when
227 increasing the volume of extracted eDNA screened (Fig. 2). The uncertainty decreased differently
228 between the three species, however, all species reached zero uncertainty (i.e., detection rate never
229 changes between outcomes) when screening at least 14 µL of extracted DNA.

230 We detected a contamination (a confirmed amplicon of the targeted species) in one negative
231 filtration control for *A. fluviatilis* on the first sampling day. From the total 16 µL of extracted
232 DNA, the negative filtration control screened showed a positive amplicon in three PCR replicates
233 (i.e., 6 µL of screened DNA). Attempts to measure DNA concentration from this negative
234 filtration control failed as DNA concentrations were too low (limit of detection reported for Qubit
235 high sensitivity assays < 0.001 µg/mL). All other negative controls (filtration, extraction and
236 PCR) were blank. The three positive amplifications of this negative filtration control happened
237 within replicates in the same PCR set up, thus we are confident that the contamination happened
238 during the preparation of the PCR reactions, and was not a contamination from the field.
239 Unfortunately, due to the testing of the extractions for so many replicates and species, we ran out
240 of the extracted DNA before being able to repeat this PCR. In order to be most conservative, we
241 excluded the data for this species from the first day from all analyses.

242 **Discussion**

243 Comparing the effect of sampled water volume and an eDNA based detection across three
244 macroinvertebrate species, we surprisingly found that only one species (*Gammarus pulex)* had a
245 positive relationship with an increased detection rate when more water was sampled. The
246 detection rate of the other two species did not correlate with sampled water volume. Based on the

247 results of a previous study using the same primers[21], we calculated the detection probability (i.e.,
248 the detection with eDNA divided by the proven presence with traditional monitoring method) in
249 river systems using the same primers. This detection probability was 0.83 for *A. fluviatilis*, 1 for
250 *B. buceratus* and 0.71 for *G. pulex*. Thus, as the latter species seems to have in general a lower
251 detectability, it may be the one most affected by the total amount of water volume sampled.
252 Indeed, we found a dependence of water volume and detection rate for this species at the lower
253 volumes, but the effect leveled off above 1 L, and then saturated at the species' overall detection
254 probability (i.e., detection rate at 1 L = 0.72 ± 0.18 and at 2 L = 0.75 ± 0.15). Surprisingly we did
255 not find a positive relationship between water volume and detection rate for all three species. We
256 speculate that such a relationship exists also for the other two species. We think that we did not
257 reach the lower limit of the detection where this relationship exists for *A. fluviatilis* and *B.*
258 *buceratus*, while for *G. pulex* we were within the water volume range where the saturation
259 occurs. Our results thus indicate that detection rates may vary by species and volume, which
260 should be considered when designing targeted eDNA detection tool.

261 All species' DNA was detected in at least one PCR replicate at the smallest volume of 250
262 mL. The detection rate had a lower uncertainty at higher volumes of DNA extraction screened
263 compared to lower volumes (Fig. 2) and indicates that screening more of the DNA extraction
264 allows for a smaller uncertainty in detection rate for a species with the used protocols across any
265 volume. The volume of extraction we screened is similar to other studies using filtration as DNA
266 capturing approach[27-29], and these studies sampled similar volumes (0.2 to 1 L) of water (Table
267 1). Even when we sampled two liters of water, we found in none of the three species studied a
268 positive signal in 100% of the PCR replications. PCR is a stochastic process and, subsequently,
269 PCR replication (e.g., the volume of screened eDNA) cannot be neglected even when sampling
270 larger volumes. In Fig. 2 we illustrate the importance of screening enough volume of extracted

DNA to decrease the uncertainty in the outcome of the detection. Although this is intuitive, it is an important aspect to keep in mind when creating a protocol for species detection with eDNA. We recommend testing volume dependence for each species that should be detected with eDNA, so that eDNA protocols are optimized with respect to sampling volume and screened volume of eDNA extractions. When resources are limited, we suggest maximizing the volume of extracted eDNA which is screened and not necessarily to maximize the water volume sampled. PCR replication is more cost effective than the filtration and extraction of larger volumes of water due to two reasons: first, at our study site we were not able to filter more than 250 mL on one single glass fiber filter because of free floating particles that clog the filters, even though we conducted our work in a river which is known to have an overall low level of particle and sediment load. For filtering volumes of two liters, we needed up to eight filters. The usage of multiple filters for the same sample is a time consuming and costly step, as each filter costs about 1 USD. Second, the extraction will be even more time intense, as each of the filters needs to be extracted separately due to limitations of tube sizes and limited ability to handle large volumes (greater than 2 L) for most standard molecular genetic laboratory centrifuges. One may need to validate these results across various river systems, in order to adjust methods for different eDNA quality and degradation, primer sensitivity and sediment loads. However, the conditions in the river Glatt are likely valid for rivers and streams in human-modified temperate landscapes with a mixed land-use of urban areas, forests and agricultural land-use.

We especially want to highlight the relevance of negative controls. We performed negative controls during three steps in the handling process: for filtration, extraction and PCR. Contaminations can occur, especially while filtering in the field, but through a good study design it is possible to track down the source of contaminations. We suggest that it is relevant to not only screen a certain volume of extracted DNA but also to screen an adequate volume for the

295 negative filtration controls. In our study we had contamination for one species in one filtration
296 control for the first day. As all the positive amplicon showed up in one set of PCR it is very likely
297 that that our contamination happened during the preparation of the PCR. However, as we cannot
298 rule out field contamination, we were stringent and have excluded all detections of this species
299 from the first day. The implication of excluding dubious data is especially important for
300 environmental DNA work were low amounts of DNA are handled and small contaminations can
301 lead to false positives. Given our findings regarding the effect of volume (either from the
302 environment or from the extraction) on uncertainty in detection rates we especially encourage
303 eDNA researchers to report the number and volume of negative controls that are screened to
304 ensure accountability. When this tool becomes used in controversial cases it will be paramount to
305 uphold the same practices and standards we apply to samples also to negative controls for proper
306 inference.

307 Optimization of single species detection through eDNA may depend on the specific
308 species and environmental settings; however, some critical considerations and guidelines can still
309 be inferred from our results. Overall we find that there may be different factors contributing to
310 the successful detection of species using eDNA. First, we conclude based on first principles that
311 at a given concentration of eDNA molecules in the environment, there must be a lower volume
312 threshold at which detection becomes less likely, while the detection rate saturates at higher
313 volume due to other factors (e.g., primer performance[12] and competition of target DNA vs. non-
314 target DNA during PCR[21]). Our data, however, suggest that this level is below 250 mL of water,
315 at least for the three species tested here and under this molecular workflow. While increasing
316 sampling volume may indeed be beneficial in reducing false negative detections (i.e., reducing
317 uncertainty in detection rate in our study) for some species, increasing the volume of extracted
318 DNA screened and primer performance may become more important due to a lower detection

rate for smaller environmental sampling volumes. Second, when comparing methods, one not only needs to quantify detection thresholds and rates of false negatives for the eDNA method, but also the method it is compared with. While the issue of both false positives and false negative detections has been raised for eDNA-approaches[2,9], it is often ignored for traditional methods, where a perfect sampling is often implicitly assumed (but see[8,30,31] on extensive discussion on this topic). Based on our results we can give a rough recommendation that sampling at least 1 L and screening a minimum of 14 µL of total extracted DNA should reduce false negative detections particularly for macroinvertebrates in freshwater and potentially for other macro-species surveillance. The recommendations should be taken with some precautions as results might be changing even within similar species and environments. We caution researchers to carefully plan sampling designs with regard to volume and encourage pilot testing these parameters in order to maximize potential detection rates for other systems.

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References

(1) Lawson Handley, L. How will the 'molecular revolution'contribute to biological recording? *Biological Journal of the Linnean Society.* **2015**, *115*, (3), 750-766.

(2) Thomsen, P. F.; Willerslev, E. Environmental DNA–an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation.* **2015**, *183*, 4-18.

(3) Goldberg, C. S.; Strickler, K. M.; Pilliod, D. S. Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation.* **2015**, *183*, 1-3.

(4) Kelly, R. P.; Port, J. A.; Yamahara, K. M.; Martone, R. G.; Lowell, N.; Thomsen, P. F.; Mach, M. E.; Bennett, M.; Prahler, E.; Caldwell, M. R. Harnessing DNA to improve environmental management. *Science.* **2014**, *344*, (6191), 1455-1456.

(5) Roussel, J. M.; Paillisson, J. M.; Tréguier, A.; Petit, E. The downside of eDNA as a survey tool in water bodies. *Journal of Applied Ecology.* **2015**, *52*, (4), 823-826.

(6) Rees, H. C.; Maddison, B. C.; Middleditch, D. J.; Patmore, J. R.; Gough, K. C. The detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology.* **2014**, *51*, (5), 1450-1459.

(7) Jerde, C. L.; Mahon, A. R. Improving confidence in environmental DNA species detection. *Molecular Ecology Resources.* **2015**, *15*, (3), 461-463.

(8) Haase, P.; Murray-Bligh, J.; Lohse, S.; Pauls, S.; Sundermann, A.; Gunn, R.; Clarke, R., Assessing the impact of errors in sorting and identifying macroinvertebrate samples. In *The Ecological Status of European Rivers: Evaluation and Intercalibration of Assessment Methods*, Springer: 2006; pp 505-521.

(9) Schmidt, B. R.; Kéry, M.; Ursenbacher, S.; Hyman, O. J.; Collins, J. P. Site occupancy models in the analysis of environmental DNA presence/absence surveys: A case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution.* **2013**, *4*, (7), 646-653.

(10) Deiner, K.; Walser, J.-C.; Mächler, E.; Altermatt, F. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation.* **2015**, *183*, 53-63.

(11) Eichmiller, J. J.; Miller, L. M.; Sorensen, P. W. Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular ecology resources.* **2015**.

(12) Wilcox, T.; McKelvey, K.; Young, M.; Jane, S.; Lowe, W.; Willson, R. C. Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *PLoS One.* **2013**, *8*, (3), e59520.

(13) McKee, A. M.; Spear, S. F.; Pierson, T. W. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation.* **2015**, *183*, 70-76.

(14) Schnell, I. B.; Bohmann, K.; Gilbert, M. T. P. Tag jumps illuminated–reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources.* **2015**, *15*, (6), 1289-303.

(15) Esling, P.; Lejzerowicz, F.; Pawlowski, J. Accurate multiplexing and filtering for high-throughput amplicon-sequencing. *Nucleic Acids Research.* **2015**, *43*, (5), gkv107.

(16) Gotelli, N. J. A primer of ecology. Sunderland, Massachusetts. In Sinauer Associates, Inc: 2001.

(17) Turner, C. R.; Uy, K. L.; Everhart, R. C. Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation.* **2015**, *183*, 93-102.

(18) Schill, W. B.; Mathes, M. V. Real-time PCR detection and quantification of nine potential sources of fecal contamination by analysis of mitochondrial cytochrome b targets. *Environmental science & technology.* **2008**, *42*, (14), 5229-5234.

(19) Goldberg, C. S.; Pilliod, D. S.; Arkle, R. S.; Waits, L. P. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS One.* **2011**, *6*, (7), e22746.

(20) Deiner, K.; Altermatt, F. Transport distance of invertebrate environmental DNA in a natural river. *PloS one.* **2014**, *9*, (2), e88786.

(21) Mächler, E.; Deiner, K.; Steinmann, P.; Altermatt, F. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. *Freshwater Science.* **2014**, *33*, (4), 1174-1183.

(22) AWEL *Zürcher Gewässer 2012: Entwicklung - Zustand - Ausblick.*; AWEL Amt für Abfall, Wasser, Energie und Luft, Zürich.: 2012.

(23) Altermatt, F.; Seymour, M.; Martinez, N. River network properties shape α-diversity and community similarity patterns of aquatic insect communities across major drainage basins. *Journal of Biogeography.* **2013**, *40*, (12), 2249-2260.

(24) Altermatt, F.; Alther, R.; Fišer, C.; Jokela, J.; Konec, M.; Küry, D.; Mächler, E.; Stucki, P.; Westram, A. M. Diversity and Distribution of Freshwater Amphipod Species in Switzerland (Crustacea: Amphipoda). *PLoS One.* **2014**, *9*, (10), e110328.

(25) Team, R. C. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. In ISBN 3-900051-07-0: 2014.

(26) Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823.* **2014**.

(27) Eichmiller, J. J.; Bajer, P. G.; Sorensen, P. W. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS One.* **2014**, *9*, (11), e112611.

(28) Spear, S. F.; Groves, J. D.; Williams, L. A.; Waits, L. P. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation.* **2015**, *183*, 38-45.

(29) Takahara, T.; Minamoto, T.; Doi, H. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS One.* **2013**, *8*, (2), e56584.

(30) Kery, M. Inferring the absence of a species: a case study of snakes. *The Journal of wildlife management.* **2002**, *66*, (2), 330-338.

(31) Kery, M.; Plattner, M. Species richness estimation and determinants of species detectability in butterfly monitoring programmes. *Ecological Entomology.* **2007**, *32*, (1), 53-61.

(32) Kortbaoui, R.; Locas, A.; Imbeau, M.; Payment, P.; Villemur, R. Universal mitochondrial PCR combined with species-specific dot-blot assay as a source-tracking method of human, bovine, chicken, ovine, and porcine in fecal-contaminated surface water. *Water research.* **2009**, *43*, (7), 2002-2010.

(33) Jerde, C. L.; Mahon, A. R.; Chadderton, W. L.; Lodge, D. M. "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters.* **2011**, *4*, (2), 150-157.

(34) Minamoto, T.; Yamanaka, H.; Takahara, T.; Honjo, M. N.; Kawabata, Z. i. Surveillance of fish species composition using environmental DNA. *Limnology.* **2012**, *13*, (2), 193-197.

(35) Olson, Z. H.; Briggler, J. T.; Williams, R. N. An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildlife Research.* **2012**, *39*, (7), 629-636.

(36) Takahara, T.; Minamoto, T.; Yamanaka, H.; Doi, H.; Kawabata, Z. i. Estimation of fish biomass using environmental DNA. *PloS one.* **2012**, *7*, (4), e35868.

(37) Tambalo, D. D.; Boa, T.; Liljebjelke, K.; Yost, C. K. Evaluation of two quantitative PCR assays using Bacteroidales and mitochondrial DNA markers for tracking dog fecal contamination in waterbodies. *Journal of microbiological methods.* **2012**, *91*, (3), 459-467.

(38) Thomsen, P. F.; Kielgast, J.; Iversen, L. L.; Møller, P. R.; Rasmussen, M.; Willerslev, E. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One.* **2012**, *7*, (8), e41732.

(39) Goldberg, C. S.; Sepulveda, A.; Ray, A.; Baumgardt, J.; Waits, L. P. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science.* **2013**, *32*, (3), 792-800.

(40) Jerde, C. L.; Chadderton, W. L.; Mahon, A. R.; Renshaw, M. A.; Corush, J.; Budny, M. L.; Mysorekar, S.; Lodge, D. M. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences.* **2013**, *70*, (999), 1-5.

(41) Mahon, A. R.; Jerde, C. L.; Galaska, M.; Bergner, J. L.; Chadderton, W. L.; Lodge, D. M.; Hunter, M. E.; Nico, L. G. Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled and field experiments. *PLoS One.* **2013**, *8*, (3), e58316.

(42) Pilliod, D. S.; Goldberg, C. S.; Arkle, R. S.; Waits, L. P. Estimating Occupancy and Abundance of Stream Amphibians Using Environmental DNA from Filtered Water Samples. *Canadian Journal of Fisheries and Aquatic Sciences.* **2013**, *70*, (ja), 1123-1130.

(43) Vuong, N.-M.; Villemur, R.; Payment, P.; Brousseau, R.; Topp, E.; Masson, L. Fecal source tracking in water using a mitochondrial DNA microarray. *Water research.* **2013**, *47*, (1), 16-30.

(44) Jane, S. F.; Wilcox, T. M.; McKelvey, K. S.; Young, M. K.; Schwartz, M. K.; Lowe, W. H.; Letcher, B. H.; Whiteley, A. R. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources.* **2015**, *15*, (1), 216-227.

(45) Keskin, E. Detection of invasive freshwater fish species using environmental DNA survey. *Biochemical Systematics and Ecology.* **2014**, *56*, 68-74.

(46) Pilliod, D. S.; Goldberg, C. S.; Arkle, R. S.; Waits, L. P. Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources.* **2014**, *14*, (1), 109-116.

(47) Amberg, J. J.; McCalla, S. G.; Monroe, E.; Lance, R.; Baerwaldt, K.; Gaikowski, M. P. Improving efficiency and reliability of environmental DNA analysis for silver carp. *Journal of Great Lakes Research.* **2015**, *41*, (2), 367-373.

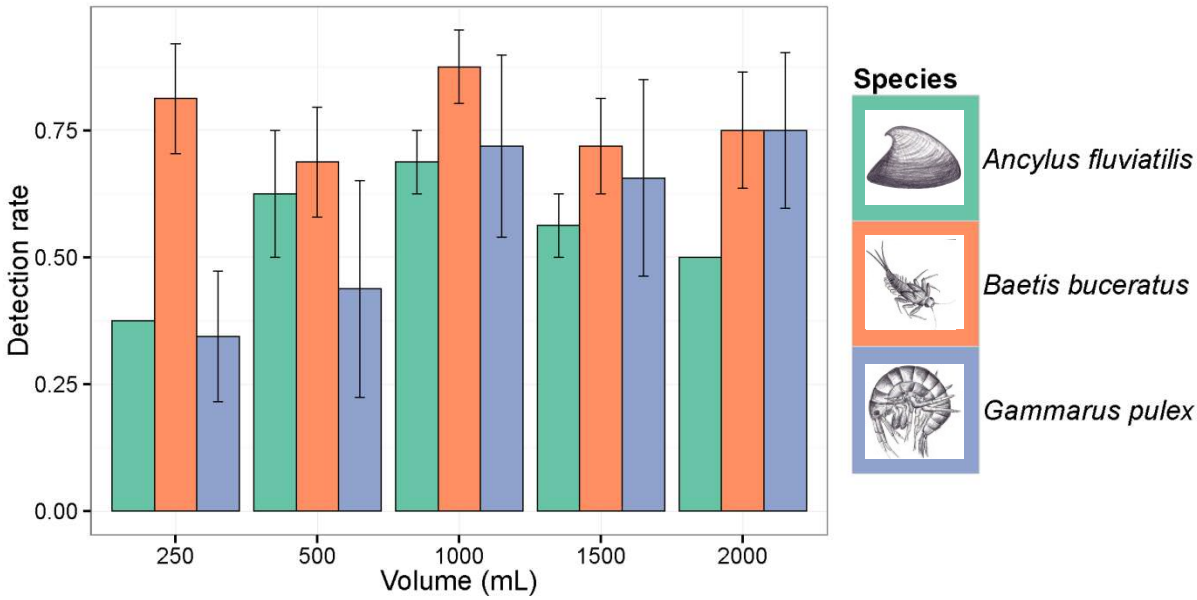
(48) Fukumoto, S.; Ushimaru, A.; Minamoto, T. A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. *Journal of Applied Ecology.* **2015**, *52*, (2), 358-365.

(49) Hunter, M. E.; Oyler-McCance, S. J.; Dorazio, R. M.; Fike, J. A.; Smith, B. J.; Hunter, C. T.; Reed, R. N.; Hart, K. M. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS One.* **2015**, *10*, (4), e0121655.

(50) Janosik, A. M.; Johnston, C. E. Environmental DNA as an effective tool for detection of imperiled fishes. *Environmental Biology of Fishes.* **2015**, *98*, (8), 1-5.

(51) Laramie, M. B.; Pilliod, D. S.; Goldberg, C. S. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation.* **2015**, *183*, 29-37.

476 **Figures**



477
478 Fig. 1: Detection rate, as proportion of positive amplifications across eight PCR replicates for
479 each volume replication, relative to water volume sampled. Error bars stand for standard errors
480 across four samples (*B. buceratus* and *G. pulex*) and two samples (*A. fluviatilis*) respectively.

481

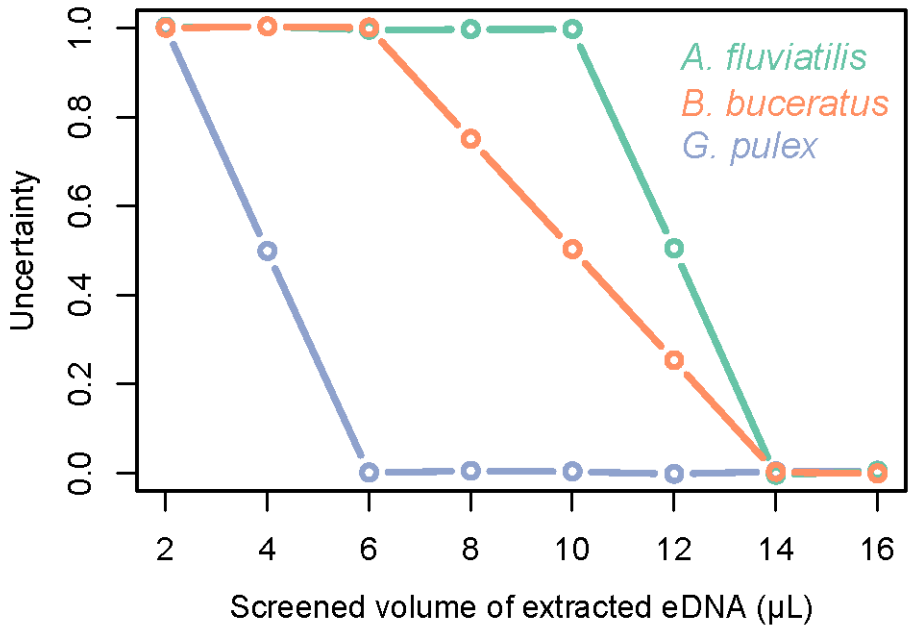


Fig. 2: Uncertainty in the detection rate relative to screened volume of extracted environmental DNA. When uncertainty is one, detection rates and thus the outcome vary between 0 and 1; when uncertainty is zero, detection rate is completely consistent between outcomes. The uncertainty of detection rate is decreasing with increasing volume of screened DNA for all three species and reaches zero at 14 µL of screened volume of eDNA.

Tables

Table 1: Overview on published methods and quantities used for filtering eDNA from macrofauna in natural aquatic systems as well as used PCR conditions. If dilution series were done the given volume for PCR template is the maximum. Samples per site are only reported if they are taken at the same sampling site, several samples that were taken within the same sampling system were not reported.

Authors	Year	Citation	Filter material	Filter pore size (µm)	Sampled water volume (mL)	Type of PCR	Template volume used for PCR (µL)	Total PCR volume (µL)	Number of PCR replications	Samples per site (e.g. volume replication)	Total PCR replication per site	Volume of extraction screened
Schill & Mathes	2008	[18]	polyethylensulfone	0.22	100-1000	real time	2.5	12.5	6	1	6	15
Kortbaoui et al.	2009	[32]	nitrocellulose	022 or 0.45	150-300	singleplex	NA	50	1	NA	NA	NA
						nested	2^{+}	50	1	NA	NA	NA
Goldberg et al.	2011	[19]	cellulose nitrate*	0.45	5000-10000	standard	1	10	1	1 (10 L), 2 (5L)	1-2	1-2
			cellulose nitrate*	0.45	5000	multiplex	1	7	6	1	6	6
Jerde et al.	2011	[33]	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Minamoto et al.	2012	[34]	polycarbon	3	2000	standard	4	25	1	1	1	4
Olson et al.	2012	[35]	glass fiber	1.5	8000	standard	1	10	10	1	10	10
Takahara et al.	2012	[36]	cellulose acetate	3	2000	quantitative	5	20	3	1	3	9
Tambalo et al.	2012	[37]	NA	0.45	500	quantitative	2-4	25	2	1	2	4-8
Thomsen et al.	2012	[38]	nylon	0.45	500	standard	2	25	8	3	24	48
Goldberg et al.	2013	[39]	cellulose nitrate	0.45	4000	quantitative	2.5	10	3-9	3	9-27	22.5-67.5
Jerde et al.	2013	[40]	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Mahon et al.	2013	[41]	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
Pilliod et al.	2013	[42]	cellulose nitrate*	0.45	1000	quantitative	2	10	3-6	1	3-6	6-12

Authors	Year	Citation	Filter material	Filter pore size (µm)	Sampled water volume (mL)	Type of PCR	Template volume used for PCR (µL)	Total PCR volume (µL)	Number of PCR replications	Samples per site (e.g. volume replication)	Total PCR replication per site	Volume of extraction screened
Schmidt et al.	2013	[9]	polyethylensulfone	NA	600	quantitative	NA	NA	2	1	2	NA
Takahara et al.	2013	[29]	cellulose acetate	3	1000	real time	2	20	8	1	8	16
Vuong et al.	2013	[43]	NA	0.45	300	standard	NA	25	NA	NA	NA	NA
						nested	1[+]	50	NA	NA	NA	NA
						quantitative	5	20	3	NA	NA	NA
Wilcox et al.	2013	[12]	glass fiber	1.5	6000	quantitative	4	20	26-32	1	26-32	104-128
Eichmiller et al.	2014	[27]	glass fiber	1.5	200	quantitative	5	25	3	1	3	15
Jane et al.	2014	[44]	glass fiber	1.5	6000	quantitative	4	20	3	1	3	12
Keskin et al.	2014	[45]	polyethylensulfone	0.22	2000	standard	5	25	3-6	3	9-18	45-90
Mächler et al.	2014	[21]	glass fiber	0.7	900	standard	2	15	8	1	8	16
Pilliod et al.	2014	[46]	cellulose nitrate*	0.45	2000	quantitative	2	10	3	1	3-6	6-12
Amberg et al.	2015	[47]	glass fiber	1.5	2000	standard	NA	25	8	NA	NA	NA
			glass fiber	1.5	2000	quantitative	1	25	8	NA	NA	8
Fukumoto et al.	2015	[48]	glass fiber	0.7	4000	real time	2	20	4	1	4	8
Hunter et al.	2015	[49]	cellulose nitrate	0.45	250-1000	quantitative	NA	20.4	3	1 - 3	9 - 27	NA
Janosik & Johnston	2015	[50]	glass fiber	1.5	2000	standard	1	25	3	NA	NA	3
Laramie et al.	2015	[51]	cellulose nitrate	0.45	1000	quantitative	3	15	3	3	9	27
McKee et al.	2015	[13]	cellulose nitrate	0.45	250-1000	quantitative	3.75	15	3	1	3	11.25
Spear et al.	2015	[28]	cellulose nitrate*	0.45	1000	quantitative	3	15	3-6	1	1	9-18

492 * Used only half filter to extract DNA

493 + Template of first PCR product

494 NA Not available from publication

Table 2: GLMM results on the effect of volume for the detection of each species.

	Coefficient	**Standard error**	**Z-value**	**p-value**
A) *Ancylus fluviatilis*				
Intercept	0.0973	0.4309	0.23	0.82
Volume	0.0001	0.0004	0.28	0.78
B) *Baetis buceratus*				
Intercept	1.5214	0.7362	2.07	0.04
Volume	-0.0001	0.0003	-0.36	0.72
C) *Gammarus pulex*				
Intercept	-1.1762	1.1871	-0.99	0.32
Volume	0.0016	0.0004	4.34	< 0.01

Abstract art